NOTE ***Public Health***

Assessment of Antibiotic Resistance Phenotype and Integrons in *Salmonella enterica* serovar Typhimurium Isolated from Swine

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ABSTRACT. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) isolated and identified from swine were subjected for the analysis of antibiotic resistance pattern and clinically important class 1 and 2 integrons. In addition, *S.* Typhimurium isolates exhibiting ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline and florfenicol (ACSSuTF) resistance pattern as described in most *Salmonella enterica* serotype Typhimurium definitive type 104 (DT104) were characterized by polymerase chain reaction. All the isolates were resistant to more than four antibiotics and showed the highest resistance to streptomycin (94.1%), followed by tetracycline (90.1%), ampicillin (64.7%), chloramphenicol (56.8%) and gentamicin (54.9%). MIC value for the ten isolates ranged between 0.125–2 μg/m*l* for ciprofloxacin. Among the beta-lactams used, only one of the isolate exhibited resistance to ceftiofur (MIC 8 μg/m*l*). Sixty eight percent of these multi drug resistance (MDR) *S.* Typhimurium isolates carried clinically important class 1 integron with 1kb (*aadA*) and/or 2kb (*dhfrXII-orfF-aadA2*) resistance gene cassettes. This study reports the increasing trend of multi drug resistance (MDR) *S.* Typhimurium with clinically important class 1 integron in pigs. In addition, emergence of the ACSSuTF-type resistance in *S.* Typhimurium PT other than DT104 may limit the use of resistance gene markers in its detection methods by PCR.
KEY WORDS: Integron, PCR, Salmonella Typhimurium.

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Salmonella enterica serotype Typhimurium is a common cause of the food borne gastroenteritis [3, 7, 8, 18]. Food producing animals like cattle, swine and poultry are found to be the potential reservoirs of these non-typhoidal *Salmonella*. *Salmonella* may be contracted by direct contact through these farm animal reservoirs or through animal food products [3, 7, 8]. Though, non-typhoidal salmonellosis itself is self limiting disease, it may be followed by systemic symptoms in children, elderly and immune compromised personals. In addition to the risk presented by *Salmonella* serotypes as potential farm and food borne hazard, the MDR property exhibited by this strain is highly alarming. Among the non-typhoidal *Salmonella*, the world wide epidemic strain DT104 has been characterized by the pentadrug (ACSSuT) resistant gene locus located in SGI-1[9, 12]. In addition to this, several other antibiotic resistance gene cassettes may be carried in a natural expression system called integron. Integrons may be located in mobile genetic elements like transposons and/or promiscus plasmid that facilitates wide dissemination of resistant genes [2]. In recent years, rapid emergence of the MDR microbes in farm animal has been of serious concern, especially in the zoonotic pathogen like *Salmonella* and *E. coli* [5, 15, 17]. This not only complicates the treatment process in animals and humans, but also actively facilitates the transfer of antimicrobial resistance determinants to other microbes.

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Although clinical cases of salmonellosis in farms may not be commonly observed, the animals may be potential carriers, and under any favorable instances, like environmental stress, production stress and other factors, the carrier state could culminate in disease. Carriers that shed the pathogen without any clinical symptoms are more liable to contaminate the local environment increasing potential public health challenges [8]. So, regular monitoring of this pathogen in farms has become important from animal and public health point of view. Considering this fact, our study was focused on assessing the prevalence and antibiotic resistance profile of *S.* Typhimurium isolated from different swine farms in Korea. We performed antibiogram for all these isolates and analyzed the clinically important class 1 and 2 integrons. In addition, for the *S.* Typhimurium isolates exhibiting ACSSuTF phenotype, PCR was performed with the set of primers previously described for the identification of DT104. The result of the PCR was further analyzed by the phage typing of these isolates.

Necropsied samples including feces, intestines, lungs and lymph nodes of pigs with mixed clinical signs of digestive and respiratory disorders were used for the isolation of *S.* Typhimurium spp. Suspect colonies, isolated from selective media incubated overnight with the necropsied samples, were selected by biochemical tests. Identification of isolates was confirmed by VITEK (Vitek system; bioMerieux, Marcy l'Etoile, France). All *Salmonella* strains were serotyped by slide agglutination and tube agglutination with *Salmonella* O and H group antisera, respectively (Difco Co., Franklin Lakes, NJ, U.S.A.). Once identified, the bacterial

cultures in TSB (Difco) were mixed with glycerin (20%) (Sigma Co., St. Louis, MO, U.S.A.) and stored at –70°C for further use. Among the isolates, only the non-duplicate *S.* Typhimurium isolate for the years 2000–2005 was included in this study.

Fifty-one *S.* Typhimurium isolates were tested with antibiotic susceptibility discs according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[4]. Antibiotic discs used in the test were purchased from Becton Dickinson (Heidelberg, Germany) and Oxoid Limited (Oxoid, Hampsire, UK). The active ingredients of the selected antibiotics were ampicillin, chloramphenicol, nalidixic acid, sulfamethoxazole, trimethoprim, tetracycline, gentamicin, amikacin, streptomycin, colistin, florfenicol, ceftiofur, enrofloxacin, ciprofloxacin, cefotaxime, ceftazidime and ceftriaxone. MICs of florfenicol, ceftiofur and ciprofloxacin were determined by micro broth dilution method for the strains showing complete or decreased zone diameter. *Escherichia coli* ATCC 25922 and *S.* Typhimurium DT104 ATCC 2501 were used for the quality control. Interpretations for antibiotics not characterized by CLSI were done as recommended by the supplier, except that the intermediate and sensitive isolates were grouped together. Zone diameter used to determine resistance for the interpretive criteria for florfenicol was ≤14 mm; similarly for intermediate if ranging from 15–18 mm, susceptible when ≥19 mm.

Six different oligonucleotide primer sets were used in the study (Table 1). All the primers were synthesized by Bioneer Co. (Daejeon, Korea). Each PCR mix totaled 50 μl: 5 μl of 10 × Ex*Taq* buffer (Mg^{2+} free), 1 μl of template DNA (DNA purification kit, Promega, Madison, WI, U.S.A.) 0.1 mM $MgCl_2$, 0.015 mM of each dNTPs, 50 pmole of each primer and 2.5 unit of *Taq* polymerase (iNtRON Biotechnology, Sungnam, Korea). After 5 min of initial denaturation at 94°C, PCR consisted of thirty-five cycles of denaturation for 30 sec at 94°C, annealing at 55–60°C for 30 sec, and extension for 30 sec-3 min at 72°C. Final extension was for 10 min at 72°C. *S.* Typhimurium DT104 ATCC 2501 and *E. coli* 96K062 with class 2 integron were used as positive control for all PCRs. The amplified PCR products with expected sizes were further cloned and transformed in pQE-UA 30 vector system (Qiagen, Valencia, U.S.A.) and competent *E. coli* M15 cells (Qiagen) respectively, using the manufacturer's protocol. Sequence of the plasmid DNA was determined by a dye-termination sequencing system using an automatic sequencer (Macrogen Co., Seoul, Korea). The nucleotide sequences of the amplicons were analysed using the BLAST programs of the National Center for Biotechnology Information (NCBI).

To substantiate the result of the PCR, phage typing was performed for the isolates that amplified DT104 specific gene and also for the isolates exhibiting ACSSuTF resistance phenotype in accordance with the guidelines provided by the Public Health Laboratory Service (PHLS, London, UK). Typing phages (29 species), and standard type strains (10 species) were used for the identification of the phage types. The experiment was performed twice using *S.* Typhimurium DT104 ATCC 2501, DT STm1, and DT STm193 for quality control.

Mixed broth culture mating was performed to assess the transferability of the florfenicol resistant gene, as described by Kang *et al.* [10]. Transconjugants were selected on TSA plate supplemented with florfenicol (8 μg/m*l*) and rifampicin (100 μg/m*l*), and transfer of the *floR* gene was confirmed by PCR. The transfer frequency was calculated as number of transconjugants per recipient.

Antimicrobial susceptibility: All isolates were resistant to more than 4 antibiotics used in disk diffusion test. Isolates showed the highest resistance to streptomycin (94.1%) followed by tetracycline (90.1%), ampicillin (64.7%), chloramphenicol (56.8%) and gentamicin (54.9%). Among the isolates, five of them showed ACSSuTF resistance pattern. Florfenicol MIC values for these were 32 μg/m*l* (3 isolates) and 64 μg/m*l* (2 isolates). Ten (19.6%) isolates showed the decreased zone diameter (<15 mm) for ciprofloxacin with MIC value ranging between 0.125–2 μg/m*l*. Only one isolate showed resistance to the ceftiofur in disk diffusion test with MIC 8 μg/m*l*.

Determination of integrons associated resistance genes and S. Typhimurium DT104: Thirty-five (68.6%) of the isolates amplified *int1* primer specific to class 1 integron.

Table 1. List of primer sequences used in the study

Primers	Sequence (5'–3')	Expected size (bp)	PCR annealing temp.	Reference/ Accession No.
intI1-F	5'-CCTCCCGCACGATGATC-3'	280	55	AY123253
intI1-R	5'TCCACGCATCGTCAGGC-3'			
5'CS	5'-GGCATCCAAGCAGCAAG-3'	Variable	55	AY220520
3'CS	5'-AAGCAGACTTGACCTGA-3'			
intI2-F	5'-TTATTGCFGGGATTAGGC-3'	233	50	M11277
intI2-R	5'-ACGGCTACCCTCTGTTATC-3'			
floR-F	5'-CAGGTTGAGCCTCTATATGG-3'	881	60	AY517519
floR-R	5'-ATGCAGAAGTAGAACGAGAC-3'			
Pse-1-F	5'-AATGGCAATCAGCGCTTCCC-3'	565	60	AY339985
Pse-1-R	5'-GGGGCTTGATGCTCACTCCA-3'			
DT104-F	5'-GTCAGCAGTGTATGGAGCGA-3'	162	60	Pritchett *et al.*
DT104-R	5'-AGTAGCGCCAGGACTCGTTA-3'			

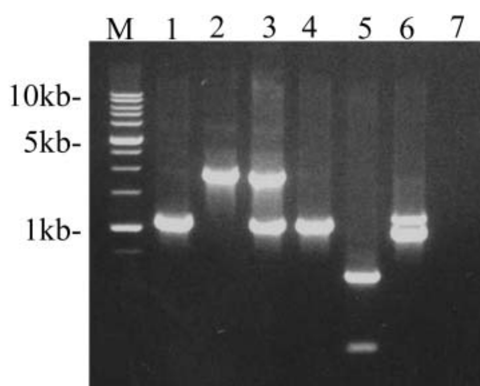

Fig. 1. PCR amplification of class 1 integron associated gene cassettes along with the *floR*, *pse-1*, and DT104 specific gene. Lane M; 1 kb DNA size marker, lanes 1, 2, 3: *S.* Typhimurium field isolates showing antibiotic resistant gene cassettes 1 kb, 2 kb and both 1 kb and 2 kb amplicons, lane 4: *floR* gene, lane 5: *pse-1* and DT104 specific gene, lane 6: 1 kb (*aadA1*) and 1.2 kb (*Pse*-1) gene cassettes amplified from the DT104 ATCC 2501 used as positive control, lane 7: PCR mix with no template.

Additional PCR of this isolates with 5CS and 3CS primer amplified two gene cassettes of 1kb (*aadA1*) and 2kb (*dhfrXII-orfF-aadA2*) within class 1 integron in three different patterns (Fig. 1, Table 2). None of the isolates amplified *intl* 2 (class 2 integrase gene) and *Pse1* gene. Similarly, only one isolate amplified DT104 specific gene as described by Pritchett *et al.* [16]. The nucleotide sequences of these amplicons were analyzed using the BLAST programs of the National Center for Biotechnology Information (NCBI).

Determination of the phage type: Phage typing of the isolate that amplified the DT104 specific gene including other four isolates exhibiting ACSSuTF resistance showed the lyses pattern of U302, DT193, and DT120 (Table 3). None of these were identified as DT104. PT U302 exhibited the phage lyses pattern very close to DT104 and showed minor differences in lyses pattern with few critical phages.

Conjugal transfer of the resistance gene: Florfenicol resistance phenotype and genotype were transferred by two isolates exhibiting ACSSuTF type resistance and identified as DT193 in phage typing at the transfer frequency of 2.0×10^{-5}. The transfer of the gene was confirmed by PCR and MIC determination of the transconjugants.

Increased resistance in microbes of animal sectors has been observed in the recent years due to the extensive use of antimicrobials in different forms. Reports of the annual survey on antibiotics for livestock 2005 has revealed the yearly average consumption of 1,450 tons of antimicrobial agents since 2001 [13]. More than half of these antibiotics were used as feed additives and for therapeutic purpose. The data give the possible explanation of high resistance to tetracycline (90.2%) and other antibiotics like ampicillin (64.7%) and gentamicin (54.9%) among these isolates.

Quinolones group of antibiotics is of special interest in treating Gram negative infections in poultry and swine industries. Our study showed increase in the percentage of quinolone resistant isolates (enrofloxacin 12.9% and nalidixic acid 47%). Like wise, the number of the ciprofloxacin

Table 2. Relationship between amplicon sizes, resistance genes, and resistance patterns of *S.* Typhimurium isolated from pigs

Groups	Amplicon size	Resistance gene	Types of resistance	No. of isolates
I	1 kbp	*AadA1*	AM-S-TE	1 (2.0%)
II	2 kbp	*dhfrXII-orfF-aadA2*	AM-C-G-S-TE NA-SXT	13 (25.5%) 9 (17.6%)
III	1&2 kbp	*aadA & dhfrXII-orfF-aadA2*	S-G-NA-SXT AM-C-TE	8 (15.7%) 4 (7.8%)
IV	No amplicon		SXT-G-S AM-C S-TE	4 (7.8%) 5 (9.8%) 7 (13.7%)

Table 3. Genetic profile and phage typing of MDR ACSSuTF-type *S.* Typhimurium isolated from pigs

Isolates No:	Genetic profile					Phage type
	aadA1	*floR*	*pse-1*	*dhfrXII-orf-aadA2*	*DT104*[a)]	
13	–	+	–	–	–	193
14	–	+	–	–	–	193
15	–	+	–	–	–	193
24	–	+	–	+	+	302
44	–	+	–	–	–	120
ATCC 2501	+	+	+	–	+	DT104

a) Pritchett *et al.*

resistant (MIC 0.125–2 μg/m*l*) isolates also showed the increased pattern compared to the previous report [18]. These observations are in agreement with the increasing use of quinolones in swine farms [13]. Among the third generation cephalosporins tested, only one isolate showed resistance to the ceftiofur with MIC (8 μg/m*l*).

The highest resistance was observed for the streptomycin (94.1%) among the antibiotics tested. Interestingly, streptomycin/spectinomycin resistance gene cassettes (*aadA1* and *aadA2*) were found to be the most frequent in the variable region of class 1 integrons (Table 2). The similar predominant pattern of this *aadA* gene in class 1 integron has been reported in microbes from other species, suggesting that this gene may be either the first cassette to be acquired by an integron and/or may be more stably integrated into the integron than other gene cassettes [2, 14].

A study on class 1 integron in *Salmonella enterica* serotype Gallinarum reported only 1.1% prevalence of trimethoprim and streptomycin/spectinomycin resistant gene (*dhfrXII-orfF-aadA2*) in the year 2002 [14]. After three years, the prevalence of the same gene was reported in 31% of the *E. coli* isolates from poultry [10]. In this study 45% of isolates amplified the same gene, indicating the increasing pattern of *dhfrXII-orfF-aadA2* gene cassettes in the conserved region of class 1 integron, by year. Recently, several other antibiotic resistance genes are reported within the conserved region of the class 1 integron [10]. This could suggest the increasing pressure of the antibiotics by year is highly associated with prevalence of antibiotic resistance and introduction of the new gene cassettes encoding resistance to antimicrobial agents commonly used. This also adds to the dynamic behavior of the class 1 integron that involves in the genetic reassortment of resistance determents [2].

In combination with ACSSuTF resistant phenotype and other genetic markers, florfenicol resistance gene (*floR*) and beta-lactam resistant gene ($bla_{pse\text{-}1}$) have been used for detection of MDR *S.* Typhimurium DT104 by PCR [1–3, 11, 18]. In this study, we tried to identify DT104 among *S.* Typhimurium pig isolates using the combination of primer sets described before [11, 16, 18]. Only one isolate exhibiting penta-drug resistance phenotype amplified the DT104 specific and *floR* gene (Table 3). However, none of the isolates amplified the *Pse-1* gene closely associated with the penta-drug resistance gene cluster of DT104. Phage typing result was in agreement with PCR assay that showed no identification of DT104 among the five ACSSuTF resistant *S.* Typhimurium isolates. The absence of the *Pse-1* gene in the *S.* Typhimurium PT U302 isolate that amplified the internal sequence of DT104 specific gene, indicated the absence of the classical construct of penta-drug resistance gene cluster in this isolate (Table 3).

Based on these observations, it could be inferred that clinically important *S.* Typhimurium PT other than DT104 could emerge with resistance to the wide spectrum of commonly used antibiotics and may exhibit penta-drug resistance profile as DT104 (R-type). Thus, identification methods of *S.* Typhimurium PT DT104 based on phenotypic antibiogram profile and resistant gene markers may be confused with other emerging MDR *S.* Typhimurium PT [3, 16, 18]. Our study showed the high specificity of the internal sequence of the DT104 specific gene and indicated its usefulness in identifying the DT104 and U302 among the *S.* Typhimurim isolates which may or may not exhibit ACSSuTF-type resistance [16]. These PT have been associated with gastroenteritis and reactive arthritis in humans [3, 8, 15, 18]. In addition, recent reports on *S.* Typhimurium PT from Taiwan and Brazil indicated that these PT could be in circulation between humans and animals [3, 15].

In conclusion, this study reports the increasing number MDR and clinically important class 1 integron among *S.* Typhimurium isolated from the pigs. In spite of the low prevalence of DT104-R type among the swine isolates, ACSSuTF type resistance could emerge independently among other clinically important PT of *S.* Typhimurium. Thus, regular monitoring of antimicrobial resistance and early detection of *S.* Typhimurium PT could be helpful in implementing prevention and control measures.

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